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# Induction of Mitochondrial Dysfunction and Apoptosis in HeLa Cells by Bis-pyridinium Oximes, a Newly Synthesized Family of Lipophilic Biscations

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**ABSTRACT.** When tested on HeLa cells, bis-pyridinium oximes (BPO), a family of newly synthesized molecules whose charged pyridinium moieties are linked by a linear polymethylene chain of variable length (N = 3 to 12) have been shown to possess an inhibitory effect on cell growth and finally to provoke cell death. BPO-affected cells displayed reduced mitochondrial oxygen consumption and ATP stores and were blocked in the G1 phase of the cell cycle. Mitochondrial membrane potential, as assayed with the dye 3,3′-diexyloxacarbocyanine iodide [$DiOC_6(3)$], increased in BPO-treated cells with time of exposure. Cell growth inhibition as well mitochondrial dysfunction were observed only with derivatives having a long polymethylene linking chain ($N \geq 6$). Furthermore, the concentration of the compound eliciting such effects was inversely related to the number of methylene groups in the linking chain. None of the BPO with N = 6 to 12 modified the mitochondrial DNA content, relative to the nuclear DNA content. In BPO (N = 8 and N = 12)-treated cells, chromatin fragmentation and internucleosomal DNA cleavage occurred massively, indicating that the death mode induced by these compounds is apoptosis. The possible pathway of action and the potential pharmacological interest of these compounds are discussed. BIOCHEM PHARMACOL 53;10:1543–1552, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** bis-pyridinium oximes; HeLa cells; mitochondrial function; mitochondrial DNA content; cell cycle; apoptosis

Tethered bispyridinium oximes (BPO) [1] are dicationic, hydrophobic compounds (Fig. 1) that were shown to accumulate in isolated, respiring mitochondria [2], as other molecules having similar characteristics [3]. Their uptake is clearly Michaelien. On the one hand, the affinity of the derivatives for the organelles (as estimated from the value of $K_m$ in Michaelis formalism) arises from the Nernst term only: it is the same throughout the BPO series and is abolished when the membrane potential is collapsed. On the other hand, the transport rate through the mitochondrial membrane (measured by the maximum velocity parameter) increases with increasing length of the linking tether and hence, increasing hydrophobicity of the derivatives [2].

BPO also intercalate DNA with weak affinity ($K = 3 \times 10^{-4}\ M^{-1}$). *In vitro*, a marked preference for single-strand DNA is observed [4]. Induction of the mutated "petite" phenotype in yeast *Saccharomyces cerevisiae* correlates with BPO uptake by purified mitochondria and is in line with BPO interaction *in vitro* with DNA. No nuclear mutation is detected under the same conditions [2, 4].

The present study was designed to examine possible biological effects of BPO in HeLa cells. Oxygen consumption, ATP stores, mitochondrial membrane potential and mitochondrial DNA content were investigated in parallel with the growth, cell cycle progression and cell death of treated cells. Results showed that continuous exposure of HeLa cells to BPO bearing a linking chain with at least 6 methylene groups was toxic to these cells by triggering their apoptosis, and also altered mitochondrial metabolism. The length of the polymethylene chain of the different deriva-

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*Abbreviations:* BPO, bis-pyridinium oximes; $DiOC_6(3)$, 3,3′-diexyloxacarbocyanine iodide; PCA, perchloric acid; SSC, (0.3 M NaCl, 30 mM $Na_3$ citrate); NAO, 10-*N*-nonyl acridine orange; PI, propidium iodide; BrdUrd, bromodeoxyuridine.

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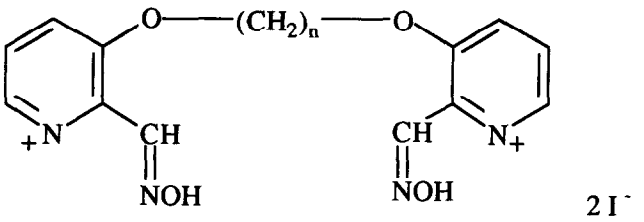


n = 3, 6, 8, 12

FIG. 1. General formula of BPO.

tives was an important parameter for their biological activity.

## MATERIALS AND METHODS

### Chemicals

BPO iodides and chlorides were synthesized and purified according to a published procedure [1].

### *Cells and Culture Conditions*

HeLa cells (ATCC CCL2), an established cell line originally derived from an adenocarcinoma of the cervix, were cultivated in Eagle's minimum essential medium supplemented with 7% fetal calf serum and 20 μg/mL gentamycin at 37°C in an atmosphere of 6% carbon dioxide and 94% air in a humidified incubator.

For the growth inhibition assay, cells were seeded in 24-well tissue culture plates in 0.5 mL of the above media. The day after (day zero), an equal volume of medium containing the BPO derivative was added to the wells. Control cells received 0.5 mL of BPO-free medium. The growth of cultures up to 6 days was followed by counting the number of cells per well after trypsinization by a Coulter counter (Coultronics, Margency, France).

### *Respiration Measurement*

After treatment with BPO, cells were rinsed twice with phosphate-buffered saline (PBS), scraped and suspended in PBS containing 2 mM glutamine and 5 mM succinate. The cell suspension was placed in a thermostatted (32°C) chamber with magnetic stirring. Oxygen consumption was measured polarographically using a Clark electrode (Yellow Springs Instrument, Yellow Springs, OH, U.S.A.) inserted into the glass vessel and connected to a recorder. Protein concentration of each sample (~2 mg/mL) was determined according to the method of Lowry *et al.* [5].

### *ATP Measurement*

Intracellular ATP content was assayed by the fluorometric method of Lowry *et al.* [6] which measures the reduction of $NADP^+$ in the presence of glucose-6-phosphate dehydrogenase, glucose, hexokinase and $Mg^{2+}$. After BPO treatment, cells were rinsed with PBS, scraped and treated with 2.6% perchloric acid (PCA). The supernatant of the pelleted PCA-treated cells after neutralization with potassium hydroxide was assayed for ATP by adding 0.2 mM $NADP^+$, 2.5 mM glucose, 5 mM $MgCl_2$, 1 E.U. hexokinase (E.C. 2.7.1.1.) and 0.5 E.U. glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49). Fluorescence emission was measured using a Perkin-Elmer LS-5 spectrofluorometer (Norwalk, CT, U.S.A.) at excitation and emission wavelengths of 334 and 456 nm, respectively. Data were normalized to the amount of proteins.

### *DNA Extraction*

DNA was extracted according to Blin and Stafford [7] with minor modifications. Briefly, at specific times after treatment, non-adherent cells were pelleted, rinsed in PBS and combined with adherent cells, also rinsed with PBS. Cells were lysed in a buffer of 10 mM Tris-HCl pH 8, 10 mM ethylenedinitrilotetraacetic acid (EDTA), 10 mM NaCl, 0.5% sodium dodecyl sulfate (SDS) containing 100 μg/mL proteinase K, incubated 12–14 hr at 37°C and extracted with phenol, phenol-$CHCl_3$:isoamyl alcohol (24:1) and $CHCl_3$:isoamyl alcohol. Nucleic acids were precipitated by ethanol, resuspended, digested by RNase (50 μg/mL) for 3 hr at 37°C and extracted again as above. After precipitation, the DNA was resuspended in 10 mM Tris-HCl (pH 7.0)-1 mM EDTA.

### *Mitochondrial DNA Quantification*

Total DNA isolated from control and treated HeLa cells was restricted with PvuII (1 unit/μg DNA, 12–14 hr, 37°C), and equal amounts (10 μg) measured spectroscopically were loaded on each lane of a 0.8% agarose gel and electrophoresed. Hybridization of DNA transferred to activated nylon membranes was performed simultaneously with two probes essentially according to Southern [8]. One probe, X1-2, was a 1.76 kb XbaI fragment of the mitochondrial ribosomal DNA partially encoding for 12S and 16S RNAs (kindly provided by Dr. P. Lestienne, Centre Hospitalier et Universitaire, Angers, France). The other probe, HU-5, was a 7.2 kb EcoR1 fragment containing nuclear-encoded 18S, 5S and 28S ribosomal DNA sequences (kindly provided by Dr. J. P. Bachellerie, Université Paul Sabatier, Toulouse, France). Probes were prepared by a random primer labeling kit (Amersham, Little Chalfont, UK), employing $\alpha[^{35}S]$-dCTP, and were purified on Sephadex G-50 (Pharmacia biotech, Uppsala, Sweden) columns. Membranes were hybridized with approximately $10^5$ cmp/$cm^2$ of each probe for 16 hr at 65°C. Final washings were done in 0.1 × SSC and 0.1% SDS at 65°C. After autoradiography of the membrane on Hyper film TM Beta Max (Amersham), the relative content of mitochondrial DNA and nuclear DNA was measured by densitometry using a Biocom (Les Ulis, France) station.

### *DNA Fragmentation Analysis*

Electrophoretic DNA fractionation of genomic DNA from combined adherent and non-adherent cells was carried out on a 2% agarose gel using 40 mM Tris-phosphate and 2 mM EDTA, pH 8 as running buffer, followed by staining with 0.5 μg/mL ethidium bromide for visualisation; 3′ end-labeling for a microscale autoradiographic analysis of apoptotic DNA fragmentation was carried out according to Tilly and Hsueh [9].

### *Fluorescence Microscopy*

To study nuclear shapes and membrane integrity, cells grown on Petriperm dishes (Bachofer, Reutlingen, Germany) were stained for 30 min with Hoechst 33342 (0.1 mg/mL) and propidium iodide (PI) (1 μg/mL) and analysed with a Leitz Fluovert Fu inverted microscope (Leica, Rueil Malmaison, France) equipped for epifluorescence. Hoechst 33342, which stains all nuclei (blue) irrespective of membrane integrity, makes it possible to determine chromatine morphology (homogeneous or fragmented). PI stains (red) only nuclei of cells with disrupted membrane. Cells with fragmented chromatine were defined as apoptotic, but according to their positive or negative staining with PI, were further classified as being either in early apoptosis or terminal apoptosis, respectively. Cells with round, homogeneously blue-stained nuclei are viable or necrotic according to a negative or positive staining with PI [10]. Quantitative analysis was performed by counting *ca.* 1000 cells in each examination.

### *Flow Cytometry*

Flow cytometry was employed to assay mitochondrial membrane potential, mitochondrial structure and cell cycle distributions. Different groups of 25 $cm^2$ flasks were seeded with $2.10^6$ HeLa cells one, two or three days before flow cytometric analysis. The day after seeding, cells were treated with the appropriate concentration of the different BPO. All groups were analyzed the same day. After rinsing with PBS, the cells were detached by trypsinization, recovered with fresh medium, centrifuged for 5 minutes at 400 *g*, resuspended in PBS and aliquoted. 3,3′-diexyloxacarbocyanine iodide [$DiOC_6(3)$] (Molecular Probes, Eugene, OR, U.S.A.) was employed as a mitochondrial probe [11]. At the concentration of 0.1 μM (with an incubation of 30 min at 37°C) employed here, this dye is an exclusive marker of mitochondrial membrane potential [12]. Moreover, at this low concentration, no toxic side-effects on the respiratory chain or the $F_0F_1$-ATPase function are observed [13]. In flow cytometry, $DiOC_6(3)$ has been used as a mitochondrial membrane potential probe in isolated mitochondria [14] and also to estimate mitochondrial dysfunction in wild-type yeast cells or in respiratory chain mutants [15] and/or in cells induced to programmed cell death [16, 17]. 10-*N*-nonyl acridine orange (NAO) (0.1 μM, with an incubation of 30 min at 37°C), a dye exhibiting a particularly high affinity for cardiolipids, was employed to estimate the eventuality of mitochondrial structure or "mass" changes induced by BPO [18, 19]. Cell viability was assayed by plasma membrane permeability using 5 μg/mL PI with an incubation of only 5 min prior to flow analysis to selectively mark dead cells.

Cell cycle analyses were done for control and BPO-treated cells pulse-labeled for 15 min at 37°C with 30 μM bromodeoxyuridine (BrdUrd) from bivariated DNA/BrdUrd distributions, according to Wilson *et al.* [20].

All cytometric measurements were performed either on a FACScan or a FACSsort (Becton-Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.) equipped with a 15 mW argon ion laser (at 488 nm) and linked to a Hewlett-Packard (Palo Alto, CA, U.S.A.) computer. Data were collected using the Becton-Dickinson Consort 30 (FACScan) or Consort 32 (FACSsort). Forward light scattering, orthogonal light scattering, and two fluorescences were measured for each sample and stored in list mode data files. The yellow-green membrane potential-related fluorescence of $DiOC_6(3)$ was recorded through a 530 ± 30 nm band pass filter using FL1 PMT, and the red fluorescence of PI was measured onto the FL3 PMT with a 650 nm long pass filter. The dead cells were electronically separated from a forward scatter vs FL3, the cells incorporating the PI being excluded from the analysis. NAO staining was followed with the same photomultiplier setting as used for $DiOC_6(3)$ [21]. The red fluorescence was recorded on the FL2 PMT (586 ± 42 nm, band pass filter) when necessary for a control but was not routinely used.

## RESULTS

### *Cell Growth Inhibition*

The effects of a series of BPO molecules, distinct from each other by the length of the aliphatic methylene chain linking the two pyridinium moieties, were tested on the growth of HeLa cells (Fig. 2). Control cultures, seeded at $3.6 \times 10^4$ cells/well, reached approximately $1.1 \times 10^6$ cells/well on the sixth day of incubation in normal medium. Addition of BPO to the medium reduced cell growth in a compound- and concentration-dependent way: the longer the linking chain, the lower the concentration of the drug eliciting cell growth inhibition and finally cell mortality. Thus, BPO with N = 3 had no effect on cell growth for concentrations up to $2.5 \times 10^{-4}$ M, BPO N = 6 and N = 8 displayed cytotoxicity between $10^{-4}$ and $3.3 \times 10^{-6}$ M and BPO N = 12 was efficient at concentrations $\geq 3.3 \times 10^{-7}$ M. It is worth noting that even for elevated compound concentrations (for example $1 \times 10^{-6}$ M of BPO N = 12) cells divided several times before being killed.

Because of their electric, hydrophobic and DNA binding properties and according to our previous *in vitro* observations [2, 4], BPO might accumulate in mitochondria of HeLa cells and hence, for example, interfere with mitochondrial energetic supply, disrupt mitochondrial structures

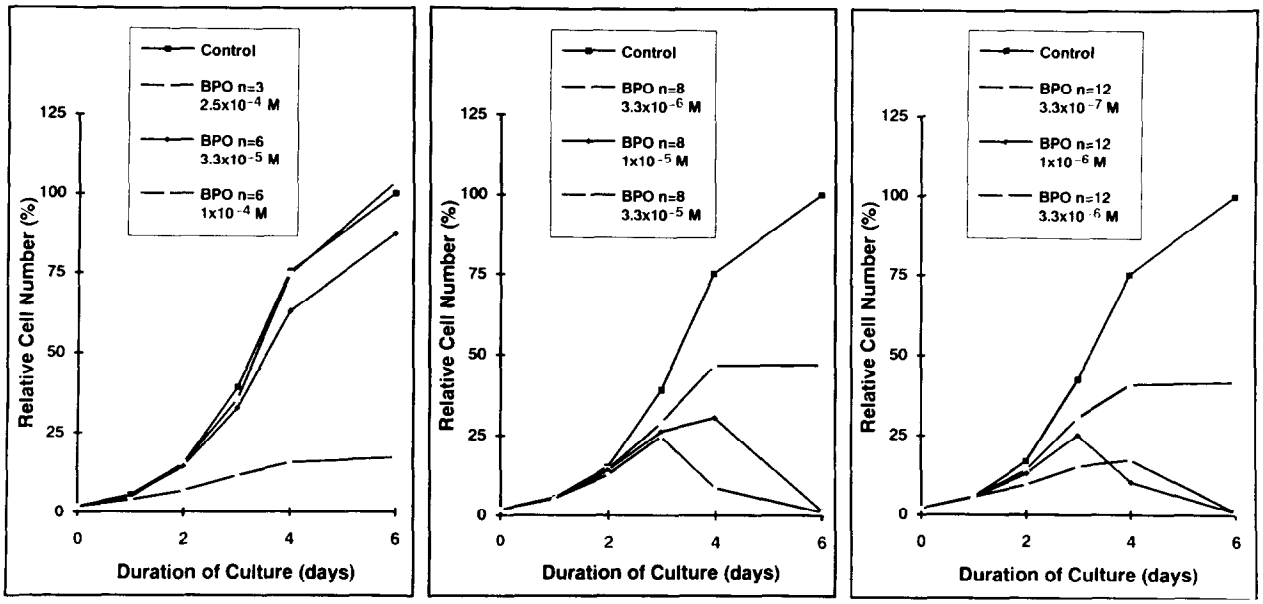


FIG. 2. Relative growth of HeLa cells incubated in presence of different BPO. Cells were initially seeded at $3.6 \times 10^4$ cells/well. One day after, BPO were added to growth medium at the indicated final concentrations. 100% value is given by the number of cells in control cultures after 6 days and corresponds to a mean of $1.1 \times 10^6$ cells/well. Data represent the means of two (BPO N = 3, BPO N = 6 and BPO N = 8) or three (BPO N = 12) separate determinations, each carried out in duplicate.

or impair mitochondrial DNA replication. BPO-treated cells were therefore analysed for their oxygen consumption, ATP stores, mitochondrial membrane potential and mitochondrial DNA content.

### *Oxygen Consumption and ATP Content*

Respiration and ATP stores in HeLa cells were measured following incubation of cultures for different time intervals with BPO N = 6 and BPO N = 12 (Fig. 3). BPO N = 6 at $10^{-4}$ M brought about only a slight reduction in respiration-mediated oxygen consumption at 24 hr. BPO N = 12 was much more effective than BPO N = 6. With the compound at the concentration of $10^{-6}$ and $3.3 \times 10^{-6}$ M, respiration was impaired after 6 hr of treatment, residual activity being ~80 and 50% of the control value. At 24 hr, oxygen consumption was more severely inhibited, dropping to ~50 and 30% of the control level.

Under identical conditions of treatment, a significant reduction in cellular ATP content was observed after 24 hr in the presence of $3.3 \times 10^{-6}$ M BPO N = 12, leading to an ATP level of ~40% of the control value. This reduction was moderate (15–20%) in all the other treated cells.

It thus seems that a decrease in oxygen consumption precedes that in ATP stores, that these impairments are slowly set up in treated cells and that they correlate with the length of the methylene chain of BPO.

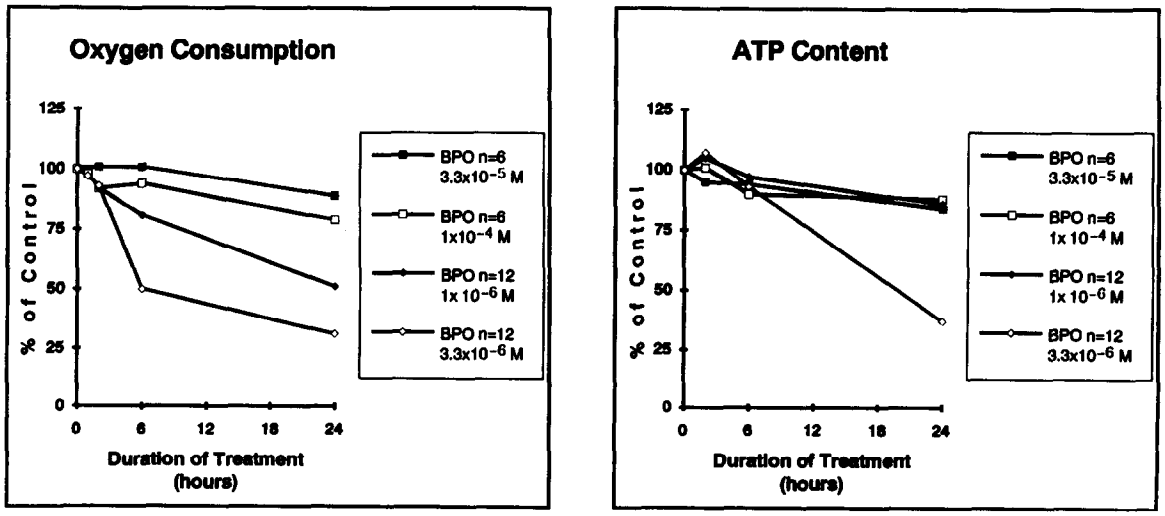


FIG. 3. Effect of incubation of HeLa cells with BPO at the indicated concentrations on cellular oxygen consumption and ATP contents. Values are averages of two independent experiments.

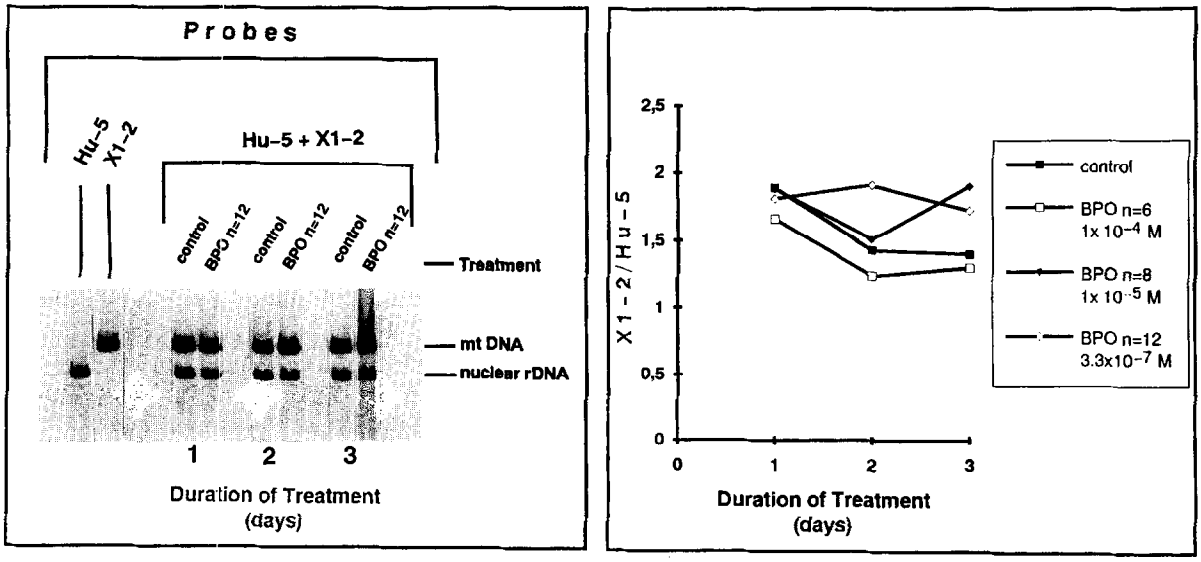


**FIG. 4. Effect of incubation of HeLa cells with BPO at the indicated concentrations on the mitochondrial DNA content of cells. Total DNA from control and treated cells was extracted, restricted with PvuII, separated by agarose gel electrophoresis, transferred according to Southern blotting and hybridized simultaneously with a mitochondrial and a nuclear ribosomal DNA radiolabeled probe. (A) Typical autoradiography: cells were treated with BPO N = 12 at $1 \times 10^{-6}$ M for the indicated period of time. (B) Quantification of another experiment in which cells were treated with different BPO derivatives for up to 3 days.**

### *Mitochondrial DNA Content*

Because of their DNA intercalating property, observed *in vitro* [4], BPO derivatives could eventually bind mitochondrial DNA *in vivo*. This could block mitochondrial DNA synthesis and result in a progressive dilution of mitochondria in successive generations of cells during the first days of treatment, or trigger some mitochondrial DNA process aimed at eliminating "damaged" mitochondrial genomes. The consequence could be a relative decrease in the number of mitochondria, and thus, of mitochondrial DNA content in treated cells.

To test this hypothesis, we measured the ratio of mitochondrial DNA to nuclear DNA in cells treated with toxic concentrations of the different BPO. As shown in Fig. 4, the mitochondrial DNA relative to nuclear ribosomal DNA remained constant in BPO-treated cells compared to control cells. This indicates that mitochondria multiply normally in treated cells and thus that BPO should not inhibit mitochondrial DNA synthesis significantly. Moreover, conformational analysis by Southern blotting under neutral and denaturing conditions of mitochondrial DNA from treated cells failed to reveal any form interconversion or degradation, indicating the absence of significant amounts of single- or double-strand DNA breaks induced by the treatment (data not shown).

### *Mitochondrial Membrane Potential*

The inhibition of oxidative phosphorylation is generally expected to diminish the electrochemical potential of mitochondria [22]. In order to assess this parameter, the lipophilic, cationic fluorochrome $DiOC_6(3)$ was applied coupled with flow spectrocytometric analysis [23].

Fluorescent cationic carbocyanines have been demonstrated to partition predominantly into mitochondria of living cells. It is assumed that the mitochondrial accumulation of these dyes is first dependent on the mitochondrial membrane potential and then on their lipophilicity. In this respect, they are often used to vitally stain mitochondria and to study their functional activity, in particular changes in membrane potential. Differential uptakes of $DiOC_6(3)$ by HeLa cells treated with BPO derivatives compared to control cells should thus reflect differences in mitochondrial membrane potential induced by the compounds.

After treatment of the cells with different concentrations of BPO for 24 or 48 hr and incubation with $DiOC_6(3)$ for 30 min, fluorescence was analysed by flow spectrocytometry. Results show that the fluorescence signal was increased in all cells treated with BPO, this increase being a function of the derivative, of its concentration and the duration of exposure of the cells. BPO N = 12 was again the most effective compound (Fig. 5A and B). It is noteworthy that in the same cells treated with BPO, the decoupling agent carbonyl cyanide *m*-chlorophenyl hydrazone led to the collapse of $DiOC_6(3)$ fluorescence, as expected when the uptake of the dye is dependent on mitochondrial membrane potential (data not shown). $DiOC_6(3)$ fluorescence was also collapsed in PI-stainable cells, i.e. having entered for the most part a late stage of apoptosis (see below).

It has been reported that membrane potential is maintained at a normal level in mitochondrial DNA-deficient cells, despite the lack of oxidatively generated ATP, by the ATP produced during the glycolytic degradation of glucose and transferred to mitochondria via the ADP/ATP translocator. Significantly, in these cells, NaF, an inhibitor of

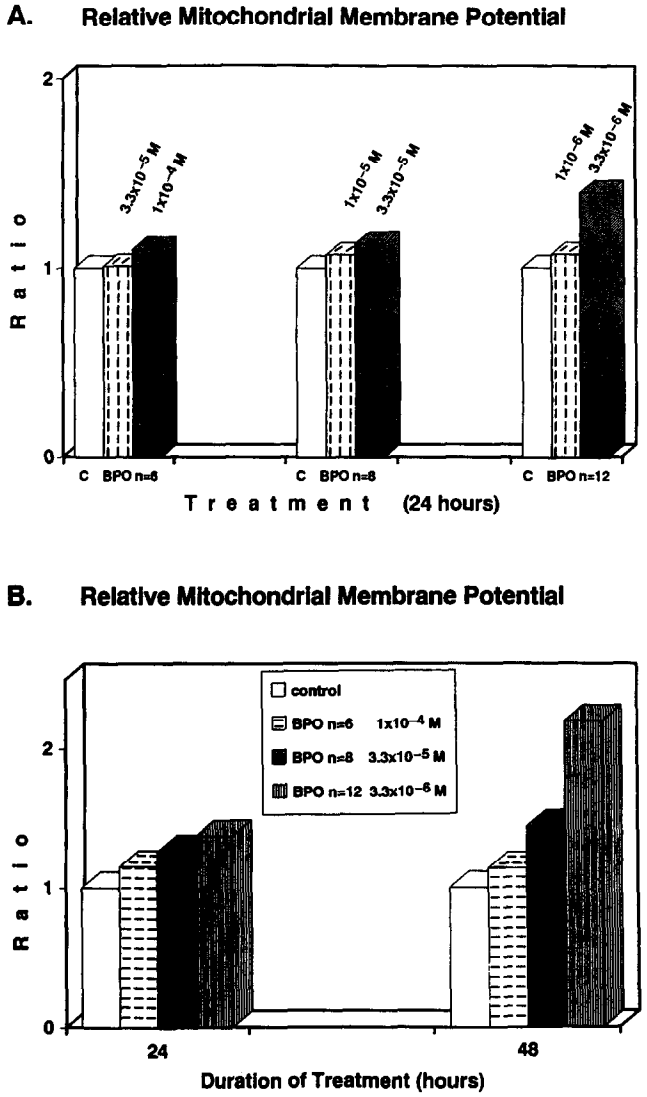


**FIG. 5. Relative mitochondrial membrane potential in BPO-treated HeLa cells. Mitochondrial membrane potential was estimated by the uptake of the cationic fluorescent dye $DiOC_6(3)$ in cells treated for 24 hr (A) or up to 48 hr (B) with BPO at the indicated concentrations and expressed relative to the uptake of untreated controls. Each point was carried out in duplicate.**

enolase, blocks glycolytic ATP generation and decreases mitochondrial membrane potential [24, 25]. We used a similar approach in HeLa cells treated with BPO N = 12 derivative. However, exposure of cells at 20 mM NaF for 2.5 hr, which was a drastic condition as judged by the considerable loss of cell viability detected in flow cytometry, had no effect on mitochondrial membrane potential as estimated by $DiOC_6(3)$-related fluorescence in BPO-treated cells. Thus, maintenance of a higher than normal membrane potential in BPO N = 12-treated cells does not seem linked to glycolytic ATP generation.

NAO fluorescence was not affected by treatment of cells with the different BPO. Since NAO interacts specifically with cardiolipids, this seems to indicate that neither mitochondrial membranes nor mitochondrial "mass" are significantly modified by BPO.

### *Cell Cycle Progression*

The consequences of a continuous exposure of cells to BPO were studied with respect to the cell cycle progression of treated cells. A flow cytometric analysis of the distribution of the cell population in the different phases of the cell cycle demonstrated a significant reduction in the cell fraction undergoing the S phase and a corresponding increase in cells in the G1 phase after 48 hr of incubation with cytotoxic concentration of BPO N = 8 and especially BPO N = 12 (Fig. 6).

### *Internucleosomal DNA Fragmentation*

The question of the mode of cell death induced by BPO was addressed. Two distinct forms of cell death, necrosis and apoptosis [26, 27], have been described, forms which differ functionally, morphologically and biochemically [28, 29, 30]. Induction of apoptosis, or programmed cell death, has been demonstrated for a number of anticancer drugs and physical agents with diverse mechanisms of action [31, 32, 33]. To assess whether BPO induce the internucleosomal DNA cleavage characteristic of apoptosis in HeLa cells, their DNA was extracted after different incubation times with BPO N = 8 or N = 12 and fractionated by electrophoresis on agarose gels (Fig. 7A). The analysis revealed a typical ladder pattern of DNA fragments in size of multiples of 180–200 base pairs in the DNA of treated cells. This pattern became evident by day 2 of treatment and further increased at day 3. No fragmentation was visible in the DNA of untreated controls. A microscale autoradiographic method based on 3′ end-labeling of DNA allowed a better visualisation of internucleosomal degradation by day 2 of treatment and confirmed that BPO are able to induce cell death of the apoptotic type (Fig. 7B).

### *Nuclear Morphology and Membrane Integrity*

To further assess the type of cell death induced by BPO, we analysed nuclear shapes and membrane integrity by fluorescence microscopy after staining with Hoechst 33342 and PI dyes. Fluorescence microscopy revealed that morphological features of apoptosis such as chromatin fragmentation and margination were clearly visible in cells by day 1 of treatment with BPO (Fig. 8). The number of altered cells increased with the drug concentration and the time of exposure (Table 1). A relatively important fraction of cells showing these typical features of apoptosis also became permeable to PI. This fraction increased with the duration of treatment, indicating a progressive loss of membrane integrity in apoptotic cells. Very few cells (<0.5%) showed a normal nuclear morphology and a staining with PI, which indicated that almost no necrosis occurs in BPO-treated cells. In most untreated cells, nuclei were homogeneously

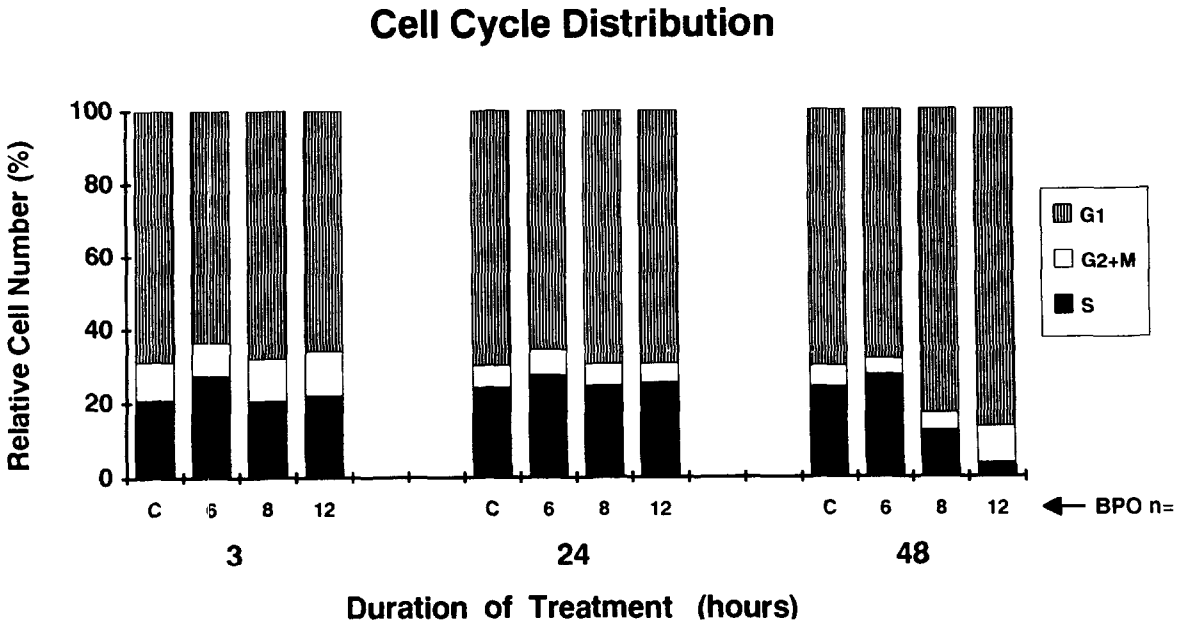


FIG. 6. Effect of incubation of HeLa cells with different BPO on cell cycle progression. Cells were treated one day after seeding for the indicated time intervals with $1 \times 10^{-4}$ M BPO N = 6 (6), $3.3 \times 10^{-5}$ M BPO N = 8 (8) or $3.3 \times 10^{-6}$ M BPO N = 12 (12). (C) represent untreated controls.

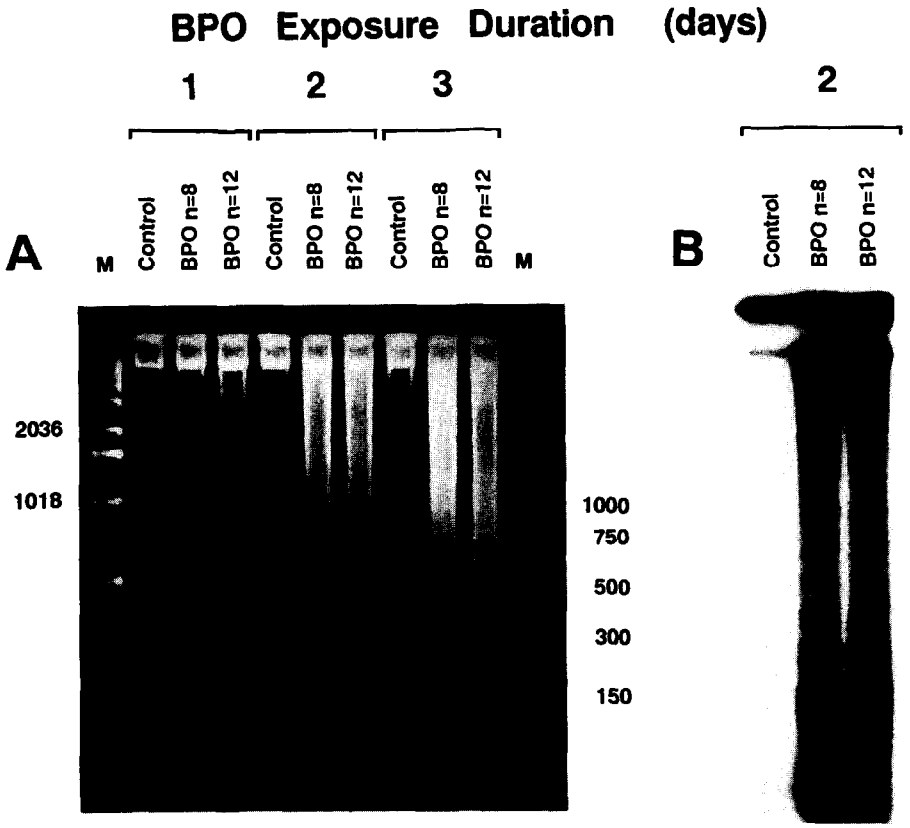


FIG. 7. Analysis of internucleosomal DNA fragmentation. (A) Agarose gel electrophoresis analysis of genomic DNA (5 μg/lane) from both non-adherent and adherent cells, untreated or treated with $3.3 \times 10^{-5}$ M BPO N = 8 or with $3.3 \times 10^{-6}$ M BPO N = 12 for 1, 2 or 3 days. M are DNA molecular weight standards whose sizes are indicated in ordonate. (B) Autoradiography of 3′ end-labeled DNA by the terminal deoxynucleotidyl transferase of DNA from control cells or cells treated with $3.3 \times 10^{-5}$ M BPO N = 8 or with $3.3 \times 10^{-6}$ M BPO N = 8 for 2 days.

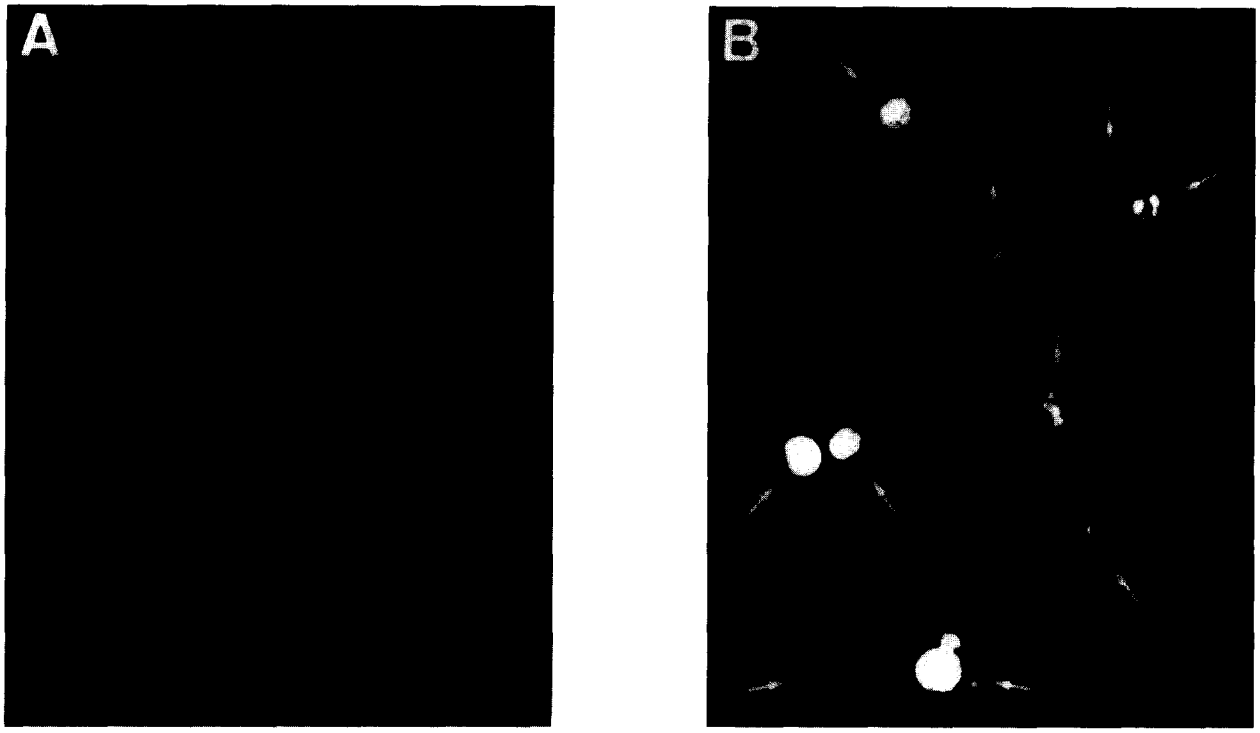


**FIG. 8. BPO induced morphological changes in HeLa cells. Cells, cultured and treated in Petriperm dishes, were stained with Hoechst 33342 and visualized by epifluorescent microscopy. Typical observations are given for (A) control cells and (B) cells incubated for 2 days with $10^{-6}$ M BPO N = 12. Arrows indicate apoptotic cells.**

stained by Hoechst 33342 at any time of culture. These results identify apoptosis as the mode of cell death induced by BPO.

## DISCUSSION

In this report, we have explored the toxic consequences of treating HeLa cells with the series of lipophilic biscations BPO. A first observation was that toxicity closely correlated with the length of the methylene chain joining the two pyridinium, i.e. with the hydrophobicity of the compound. It is likely that the ability to pass through lipid-rich membranes was an important feature for the lethal potency of these molecules. Mitochondria, because of their high negative potential and the constitution of their membranes, were thus also indicated *in vivo* as a probable target

**TABLE 1. Analysis of mode of cell death by fluorescence microscopy after treatment of HeLa cells with BPO**

| Duration of treatment (days) | Treatment | Viable cells (%) | Apoptotic cells (%) | | | Necrotic cells (%) |
|---|---|---|---|---|---|---|
| | | | Total | = Early apoptosis | + Late apoptosis | |
| 1 | None | 96.2 | 3.8 | 2.9 | 0.9 | — |
| | BPO N = 8 $1 \times 10^{-5}$ M | 93.5 | 6.4 | 3.2 | 3.2 | 0.1 |
| | BPO N = 8 $3.3 \times 10^{-5}$ M | 92.2 | 7.7 | 2.9 | 4.8 | 0.1 |
| | BPO N = 12 $1 \times 10^{-6}$ M | 91.2 | 8.7 | 3.0 | 5.7 | 0.1 |
| | BPO N = 12 $3.3 \times 10^{-6}$ M | 88.9 | 11.0 | 3.6 | 7.4 | 0.1 |
| 2 | None | 96.2 | 3.8 | 2.4 | 1.4 | — |
| | BPO N = 8 $1 \times 10^{-5}$ M | 93.0 | 7.0 | 1.8 | 5.2 | — |
| | BPO N = 8 $3.3 \times 10^{-5}$ M | 69.2 | 30.6 | 2.9 | 27.7 | 0.2 |
| | BPO N = 12 $1 \times 10^{-6}$ M | 82.4 | 17.4 | 4.6 | 12.8 | 0.2 |
| | BPO N = 12 $3.3 \times 10^{-6}$ M | 61.2 | 38.3 | 4.6 | 33.7 | 0.5 |
| 3 | None | 95.7 | 4.2 | 1.5 | 2.7 | 0.1 |
| | BPO N = 8 $1 \times 10^{-5}$ M | 54.1 | 45.7 | 3.8 | 41.9 | 0.2 |
| | BPO N = 8 $3.3 \times 10^{-5}$ M | 3.2 | 96.5 | 0.6 | 95.9 | 0.3 |
| | BPO N = 12 $1 \times 10^{-6}$ M | 20.3 | 79.5 | 8.9 | 70.6 | 0.2 |
| | BPO N = 12 $3.3 \times 10^{-6}$ M | 1.6 | 98.4 | ≈0 | ≈98.4 | ND |

Results are the mean of two independent experiments. ND: not determined.

for BPO. An attempt was thereby made to assess changes in mitochondrial functions in BPO-exposed cells. Rapid effects of the treatment were a reduction in oxygen consumption and in ATP stores. These effects preceded the blocking of cell cycle progression (characterized by an increase of the cell fraction in the G1 phase and a diminution of the cell fraction undergoing the S phase) and a significant loss of viability. At 24 hr, the oxygen consumption and ATP content of cells treated with $3.3 \times 10^{-6}$ M BPO N = 12 were decreased by approximately 70% and 60% respectively, whereas 89% of these cells were still viable (PI unstainable). Moreover, after up to three days of treatment, BPO at cytotoxic concentrations did not seem to interfere with mitochondrial DNA synthesis or to induce a significant number of DNA strand breaks in mitochondrial genome. These results indicate that, in human cells, a decrease in oxygen consumption and ATP content does not depend on loss of mitochondrial DNA or disruption of cell membrane integrity but probably occurs as a consequence of an action of BPO at the level of the respiratory chain. This appears to be at variance with what is assumed to be the mode of BPO induction of "petite" mutation in yeast [2].

Despite the induced reduction of oxidatively generated ATP, BPO apparently cause a concentration- and time-dependent hyperpolarization of mitochondrial membranes in treated cells (up to permeabilisation of their membrane in late apoptosis). Some data obtained with NaF, an inhibitor of enolase, did not support a maintenance of mitochondrial membrane potential by the ATP produced by glycolysis. On the other hand, oligomycin, an inhibitor of ATPase, has been reported to simultaneously reduce ATP production and increase mitochondrial membrane potential [34]. Thus, BPO have similar effects to oligomycin and might have a similar way of action. Tentatively, it may be supposed that proton transport across the inner mitochondrial membrane at the level of $F_0F_1$ ATPase complex is impaired by the uptake of BPO, and thus that the proton-motive force to synthesize ATP from ADP and Pi is not dissipated. Further electron transfer from NADH or FADH2 may then result in an increase in the transmembrane proton concentration gradient and in membrane electric potential. Finally, the oxidation of NADH or FADH2 will cease because of the excessive energy eventually required to pump additional protons across the inner membrane against the existing proton-motive force. It may be that as the mitochondrial membrane potential increases in cells incubated with BPO, the afflux of these molecules into the organelles is progressively facilitated. This could explain the apparent delayed cytotoxic effect of the treatment.

This persistence of high mitochondrial membrane potential in cells treated with the derivative BPO N = 12 rules out the possibility that this biscation, because of its long linking chain, could exert a detergent action able to disrupt the mitochondrial inner membrane. Moreover, the normal uptake of NAO in cells treated with BPO indicates that the import of mitochondrial protein precursors and/or the synthesis of cardiolipids of the inner membrane are not significantly affected by BPO.

Finally, morphological changes of nuclei and internucleosomal fragmentation of DNA in response to BPO exposure have indicated that apoptosis is the mode of cell death of nearly all cells killed by BPO treatment.

On the basis of these results on HeLa cells and of those previously reported on isolated organelles and yeast, it is proposed that BPO could act by penetrating energized mitochondria, inducing a dysfunction of their energetic metabolism and eliciting a programmed cell death process in exposed cells. It is noteworthy that apoptosis has already been described for different inhibitors of mitochondrial respiratory chain or of $F_0F_1$ ATPase [35]. According to this hypothesis, since it has been reported that numerous tumoral cells exhibit high mitochondrial membrane potential compared to normal cells [36, 37, 38], the BPO series could enrich the family of cationic drugs with pharmacological potential. Interestingly, the possibility of varying the hydrophobicity of BPO molecules by varying the length of their polymethylene chain could provide an easy means to modulate their cytotoxicity.

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